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Research Report

Combination of 12 % spirulina and 20 % chitosan on macrophage, PMN, and lymphocyte cell expressions in post extraction wound

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ABSTRACT

***Background**: Tooth extraction is the last treatment option for unfavorable tooth followed by dentures. Inflammation is one of phases during healing process that should be minimized to preserve alveolar bone for denture support. Macrophage, PMN and lymphocyte cells are indicators for acute inflammation. Spirulina and chitosan are natural compounds expected to be anti-inflammatory agents. **Purpose:** This research aimed to determine macrophage, PMN and lymphocyte cells of animal models treated with a combination of 12% spirulina and 20% chitosan on the 1st, 2nd and 3rd days of post extraction. **Methods:** animal models were randomly divided into control (K) and treatment (P) groups. Each group then was divided into three subgroups (KI, KII, KIII and PI, PII, PIII). The post extraction sockets of those animals in the control groups then were filled with CMC Na 3%. Meanwhile, the post extraction sockets of those animals in the treatment groups were filled with the combination of 12% spirulina dan 20% chitosan. Next, the number of PMN, macrophage and lymphocyte cells was analyzed using HE analysis on the 1st, 2nd and 3rd days. Statistical analysis then was performed using T-test. **Results:** There were a decrease in PMN cells and an increase in macrophage and lymphocyte cells on days 1, 2, and 3. **Conclusion:** It can be concluded that the combination of 12% spirulina and 20% chitosan not only can decrease PMN cells, but can also increase macrophage and lymphocyte cells on days 1, 2 and 3 after tooth extraction.*

***Keywords:** spirulina; chitosan; inflammation; PMN; macrophage; lymphocyte*

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INTRODUCTION

Tooth loss is one of dental health problems. The prevalence of tooth loss in Indonesia, according to data of RISKESDAS in 2013, was 14.51% in the age group of 45-54 years, 25.02% in the age group 55-64 years, and 43.79% in the age group of >65 years.[1] Tooth loss

can occur due to extraction process. Extraction can increase injury to dental tissues resulting in acute inflammatory reaction as well as inflammatory cell infiltration, such as polymorphonuclear (PMN), macrophages, and lymphocytes.[2] Clinical inflammatory reaction is usually indicated with edema, redness, and pain.[3] The inflammatory phase commonly lasts from a moment after the injury to day 6 after the injury.[4] Thus, to maintain the alveolar bone while setting prosthetic restoration, biomaterial is required to eliminate the inflammatory process and accelerate the healing process after the extraction.

Recently, natural materials have widely been studied for accelerating wound healing process since they are safer than synthetic materials. One of the natural ingredients that have many benefits for the wound healing process is spirulina. Spirulina is a greenish-green algae containing C-phycocyanin, B-carotenoids, vitamin E, zinc, and other components useful for the human body. C-phycocianin even is considered as an anti-inflammatory and antioxidant components.[5,6]

Another natural ingredient commonly studied for the healing process is chitosan. Chitosan is a polymer of deacetylated chitin. Chitin is a copolymer of N-acetyl-d-glucosamine and D-glucosamine bound by ß- (1-4) glycosidic bonds. Chitin and chitosan can be found in aquatic and terrestrial organisms. Chitosan can currently be obtained through food industry by processing waste derived from shrimps, lobsters, crabs, and squids.[7] Chitosan is widely used as one of ingredients for drug delivery systems, wound healing process, and orthopedic implants. Chitosan is also known to increase activities of immune cells, inflammatory cells, and angioendothelial cells. Chitosan oligosaccharides have anti-inflammatory properties since chitosan can inhibit the production of tumor necrotizing factor-α (TNF-α) in inflammation stimulated by Lipopolysaccharide (LPS).[6] Other studies have also shown that chitosan can reduce inflammation of allergic responses by inhibiting the secretion of Interleukin-8 (IL-8) and TNF-α.[7]

Some cases in the field of prosthodontics, such as immediately denture and ovate pontik installation, also require faster wound healing process. Consequently, a shorter treatment that can lead to optimal results for patient comfort is necessary. Many previous researches on effects of the induction of spirulina and chitosan on collagen, osteoblast, and osteoclast cells in model animals have been conducted. Results of the previous reseraches have shown that combination 12% spirulina and 20% chitosan not only can increase collagen and osteoblast expressions, but can also decrease osteoclast expression. As a result, it can be said that spirulina and chitosan can help the bone healing process.[8] The effects of spirulina and chitosan on bone healing process actually have already been studied, but the effects of the

combination of spirulina and chitosan on inflammatory cells have not been studied. Therefore, this research aimed to reveal effects of the combination of 12% spirulina and 20% chitosan on PMN, macrophage and lymphocyte cells in animal models on days 1, 2 and 3 after the extraction.

MATERIALS AND METHOD

This research was a laboratory experimental research with a post-test-only -control group design. This research used male Cavia cobaya animals (n = 42) weighed 300-350 grams and aged 3-3.5 months. This research has passed an ethical test performed by Faculty of Dental Medicine, Universitas Airlangga (no. 110/KKEPK.FKG/VII/2016).

Next, the animal models were divided into two groups, namely control and treatment. The control (K) and treatment (P) groups then were subdivided into three subgroups for each, namely KI, KII, KIII, PI, PII, and PIII. The roman numerals, I, II and III, indicated days 1, 2, and 3 after the animals were terminated.

Afterwards, mandibular incisors in all groups were extracted under ketamine anastesi (Ketalar, PT Pfizer, Jakarta, Indonesia) with a dose of 40 mg/kgBW. After the extraction, the sockets in the control groups, KI, KII, and KIII groups, were filled with 3% Sodium-Carboxymethyl Cellulose Natrium (CMC Na). Meanwhile, the sockets in the treatment groups, PI, PII, and PIII groups, were filled with the combination of 3% CMC Na, 12% spirulina, and 20% chitosan using a 0.1 cc syringe. Next, the sockets were stitched with silk threat sized 3/0. After the treatment, those animals were returned to their cage. On the 1st day, KI and PI groups were decaputated, and so on for the treatment of KII and PII groups on day 2, as well as KIII and PIII groups on day 3. Samples of mandibles were then taken and fixed.

After that, the mandibles were decekified with 2.5% nitric acid for 2 days. Thereafter, the sagittal incisive socket area of those animals was cut and soaked in a10% buffered formalin for 24 hours. Mixed preparations were then performed by using Echin Haematoxylin (HE). Next, PMN, macrophages, and lymphocytes on the 1/3 area of the sockets were observed using a light microscope (Nikon H600L®, Tokyo, Japan) with a magnification of 400x. The data obtained then were analyzed by using Komolgorof Smirnov test to analyze the data distribution, continued with Independent t-test to know differences between the groups.

RESULTS

Results of the observation on PMN, macrophage, and lymphocyte cells in the control groups and the treatment groups can be seen in Table 1. Results of HE staining in the control groups and the treatment groups, moreover, can be seen in can be seen in Figure 1. The results of HE staining illustrate that PMN had a large cell picture, a nucleus that had lobes (2-5 lobes), chromatin in condensed nucleus, and visible organelle in cytoplasm. The results of HE staining also depict that macrophages had a large cell image and cell nuclei located eccentrically and generally kidney-shaped. Cytoplasm cells, moreover, were solid and appeared to be a pink and purple granule, whereas lymphocytes had a circular or spherical nucleus cell with dark blue chromatin and a thin, light blue cytoplasm around it.

Next, before t-test analysis was performed, normality test, Kolmogorov-Smirnov test was conducted. Results of the Kolmogorov-Smirnov test showed that all data were normally distributed because p value was more than 0.05. Independent t-test then was carried out. Results of the independent t-test indicated that the p values of PMN cells between KI and PI groups, between KII and PIII groups, and between KIII and PIII groups were 0.020, 0.000, and 0.007, respectively. The results of the independent t-test also showed that the p values of macrophages between KI and PI groups, between KII and PII groups, and between KIII and PIII groups were 0.000, 0.000, and 0.001, sequentially. Meanwhile, the p values of lymphocytes between KI and PI groups, between KII and PII groups, and between KIII and PIII groups were 0.000, 0.000, and 0.055, respectively. Almost all of the independent t-test results in the control groups compared with the treatment groups indicated significant increase macrophages and lymphocytes differences ($p<0.05$), except between group PMN significant decrease ($p>0.05$). Thus, it can be said that there was no significant difference between both groups.

DISCUSSION

The presence of PMN cells is very important as an indicator of wound healing process since PMN cells are the first cells to appear in acute inflammatory phase.[9] PMN cells are cellular defenses that play an active role in destroying bacteria. PMN cells also play a role in the process of bacterial destruction through endothelial adherens, chemotaxis, phagocytosis, and killing bacteria. Besides, PMN cells are able to move actively, and in a short time can gather in large quantities in the wound area. PMN cells have a lifespan of 1-3 days in connective tissue.[10]

Another cell that plays a role in the inflammatory process is macrophage cell. Macrophage cells have several functions in the wound healing process, such as producing collagenase and elastase enzymes, generating cytokines, facilitating phagocytosis and angiogenesis processes, as well as stimulating granulation tissue formation in proliferative phase.[11] Macrophage cells in the inflammatory process can be distinguished by the origin of tissue macrophage resident and monocytes undergoing differentiation.[12] Monocytes in blood vessels will move toward inflammatory tissues due to chemotaxis because of the response to chemoattractant. Chemoattractant consists in partly of kemokin, a small protein (8-14 kDa) that regulates cell travel through interaction with a 7-transmembrane subset, G-protein pair receptor.[13]

Similarly, lymphocyte cells play a role in the inflammatory process. Lymphocytes participate in immune-mediated inflammation caused by infectious agents and non-immune inflammatory. T and B lymphocytes migrate to the inflammatory area and direct the neutrophils and other leukocytes.[14] In remodeling process when the wound is closed and the local infection has subsided, the leukocyte subtance most often in the injured tissue is T cell that acts as an adaptive immune response cell.[6]

Results of the research on day 1 showed that PMN cells in the control group were higher than in the treatment group. This occurred because phycocyanin and β-carotene contained in spirulina can decrease the production of proinflammatory cytokines, TNF-α and IL-1β. β-carotene in spirulina also has an anti-inflammatory effect through resistance to the production of nitric oxide and prostaglandin E2. Furthermore, β-carotene also inhibits the expression of INOS, COX2, TNFα and IL1β. Suppression of inflammatory mediators is due to NF-κB inhibition that inhibits nuclear translocation subunits NF-κB p65.[15]

As a result, antiinflammatory activity of spirulina can decrease the number of PMNs in the lesion site. TNF-α and IL-1β can also facilitate the movement of PMN cells to the site of the lesion, and spur the endothelial adherens.[16] It is intended that PMN cells easily pass through the gap between endothelial cells in capillary blood vessels to eliminate bacteria, so the decreasing of proinflammatory cytokines can lead to a decrease in the production of PMN cells.[16]

In addition, on the first day after the dental extraction, the average number of PMN cells in Group KI was the highest compared with the other control groups (K II and K III) and the treatment groups on the other days (P II and P III) because PMN cells had already worked actively and assembled at a large number of lesions very fast (within hours).[17] PMN cells are highly reactive to chemotactic products in the form of proteins produced by bacteria.[16]

Moreoever, the calculating result of macrophage cells on day 1 indicated that the treatment group (P I) had a higher number of macrophages than the control group (K I). This may occur because the inflammatory cells that appear immediately after the wound are not only neutrophils, but also monocytes moving into the inflammatory area even though the number of monocytes entering the wound area is not as much as the number of neutrophils.[18] The combination of 12% spirulina and 20% chitosan also contain more synthetic C-phycocyanin components functioning as an immunomodulation than when applied as the single one.[19]

Furthermore, the results of the independent t-test showed that there was a significant difference in lymphocytes between K1 and P1 groups on the 1st day post extraction. This may occur because phycocyanin pigments contained in spirulina may act as anti-inflammatory by inhibiting proinflammatory cytokines, namely TNF-α and IL-1β.[15] Chitosan also plays a role in increasing lymphocyte cells. A previous research using mice orally induced with chitosan finds that chitosan can stimulate the release of IL-10, IL-4, and TGF-β mRNA expressions in their gastric mucosa, CD3 + T lymphocytes in their spleen, as well as natural killer cells (NK) in their intestinal intraepithelial lymphocytes.[20]

In addition, the number of PMN cells in Group PII, based on the results of the 2nd day observation, was lower than those in Group KII. The decrease in PMN cells on the 2nd day was higher than on the first day due to homeostasis process where the number of PMN cells produced in the bone marrow must be balanced with the number of PMN cells having clearance that had already worked on the system in the body. PMN cells produced in the bone marrow can reach the maximum number within 24-48 hours after the injury. The process of PMN cell clearance, on the other hand, can reach its peak at 48 hours after the onset of the lesion. The number of PMN cells then will decrease as it enters the chronic inflammatory phase. Clearance can also occur when the PMN cells extravatate into the peripheral tissues. A previous research on mice revealed that PMN cells can migrate back from peripheral tissue into the bloodstream by a process, called as reverse transmigration.[16] This suggests that the extravatation of PMN cells does not necessarily lead to clearance of tissue. The clearance process then reaches its peak on the second day. Consequently, the anti-inflammatory effect of the combination of 12% spirulina and 20% chitosan will decrease the number of PMN cells on the second day, greater than on the first day.[16]

Moreover, the number of lymphocytes in Group PII, based on the results of the 2nd day observation, was higher than those in Group KII. The increase is due to spirulina able to increase the number of lymphocytes. Previous researches even have shown that spirulina has

an immune modulatory effect on lymphocytes by significantly increasing IFN-.2 production.[21] The increased lymphocyte cells in the treatment group is also due to chitosan that has biocompatible and biodegradable properties. The biodegradable properties allow chitosan to be broken down into micromolecules so easily absorbed by the body without causing toxicity that it can be used as an analgesic, antitumor, antimicrobial, antioxidant, and wound healing agent.[22] Chitosan also contains N-acetyl-D-glucosamine unit, polysaccharide similar to glucan, accelerating cytokine production to stimulate repair of affected tissue.[23]

Furthermore, the results of the 3rd day observation found that the number of PMN cells in Group PIII was lower than those in Group KIII. The number of PMN cells on the 3rd day was lower than on the 2nd day. This happened because the number of PMN cells usually will decrease from day 3 to day 7.[18.24] This reduction is needed to prevent further damage to body healthy tissue. The body responds to reduce the production of PMN cells in order not to damage other tissues because PMN cells have anti-microbial products that can damage healthy tissue of the body.

In addition, the number of macrophages in Group PIII, based on the results of the 3rd day observation, was still higher than those in Group KIII. Nevertheless, there was no significant difference in the number of macrophages between the treatment groups on day 2 and day 3 post-extraction. This may be due to the inflammatory process beginning to enter the resolution phase. In the resolution phase, there is a reduction of chemokine by the mechanism of proteolysis and chemokine sequestration.[25] Macrophages, as a result, begin to dominate in the wound area from day 3 to day 7 after the extraction.

Moreover, the number of lymphocyte cells in the treatment group, based on the results of the 3rd day observation, appeared to increase compared with the control group. However, based on the results of the independent t-test, there was no significant difference in the number of lymphocyte cells between the control group and the treatment group on day 3. Lymphocyte during chronic inflammatory process usually can reach its peak from day 5 to day 10.[15] Thus, 12% spirulina and 20% chitosan are expected to act as anti-inflammatory. This insignificant difference between the control group and the treatment group is due to the chronic inflammatory process that has begun to subside and enter the maturation process leading to regeneration.

Furthermore, phycocyanin and β-carotene substances contained in spirulina can be considered as anti-inflammatory and antioxidants that can accelerate wound healing process. Phycyocyanin, according to in vitro and in vivo previous researches using rat-fed animals, can inhibit TNF-α inflammatory cytokine secretion and act as an antioxidant.[26] It means that

antiinflammatory activity of spirulina may make TNF-α and IL-1β secretions decrease.[27] Similarly, antioxidants in β -carotene contained in spirulina can improve wound healing process.[6] Chitin and chitosan are biopolymers that have many benefits, including good biocompatibility, low toxicity, increased antibacterial activity, and accelerated wound healing process.[28] Chitosan can also stimulate PMN cells to chemotaxyize to the wound area due to the presence of IL-1, TNF-α, and bacterial products.[29]

In other words, spirulina and chitosan have a synergistic effect when combined since chitosan plays a role in drug delivery, while spirulina has a therapeutic effect. Spirulina and chitosan can also interact intermolecularly to increase mechanical resistance. Both of these materials even can work together and provide more effective benefits than if applied as a single one.

In addition, high chitosan acetylation degree will improve the hydrophobic properties of chitosan. The hydrophilic component is capable of diffusing through the chitosan polymer to the outer medium to be absorbed by the body.[22] Finally, based on the above discussion, it can be concluded that 12% spirulina and 20% chitosan combination not only can decrease PMN expression, but also increase macrophages and lymphocytes in cavia cobaya animals on the 1st, 2nd and 3rd days after tooth extraction.

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Table 1. Mean and standard deviation of PMN, macrophage, and lymphocyte expressions

No	Groups	n	PMN	Macrophages	Lymphocytes
1	KI	7	41.85±5.87	1.43±0.53	3.00±0.82
2	PI	7	32.00±3.26	5.57±1.51	12.00±2.00
3	KII	7	35.29±2.81	2.00±1.15	8.28±2.28
4	PII	7	11.71±1.60	19.71±1.98	21.43±2.37
5	KIII	7	20.57±3.69	15.57±1.72	19.14±2.41
6	PIII	7	15.14±2.41	20.57±2.70	22.43±3.31

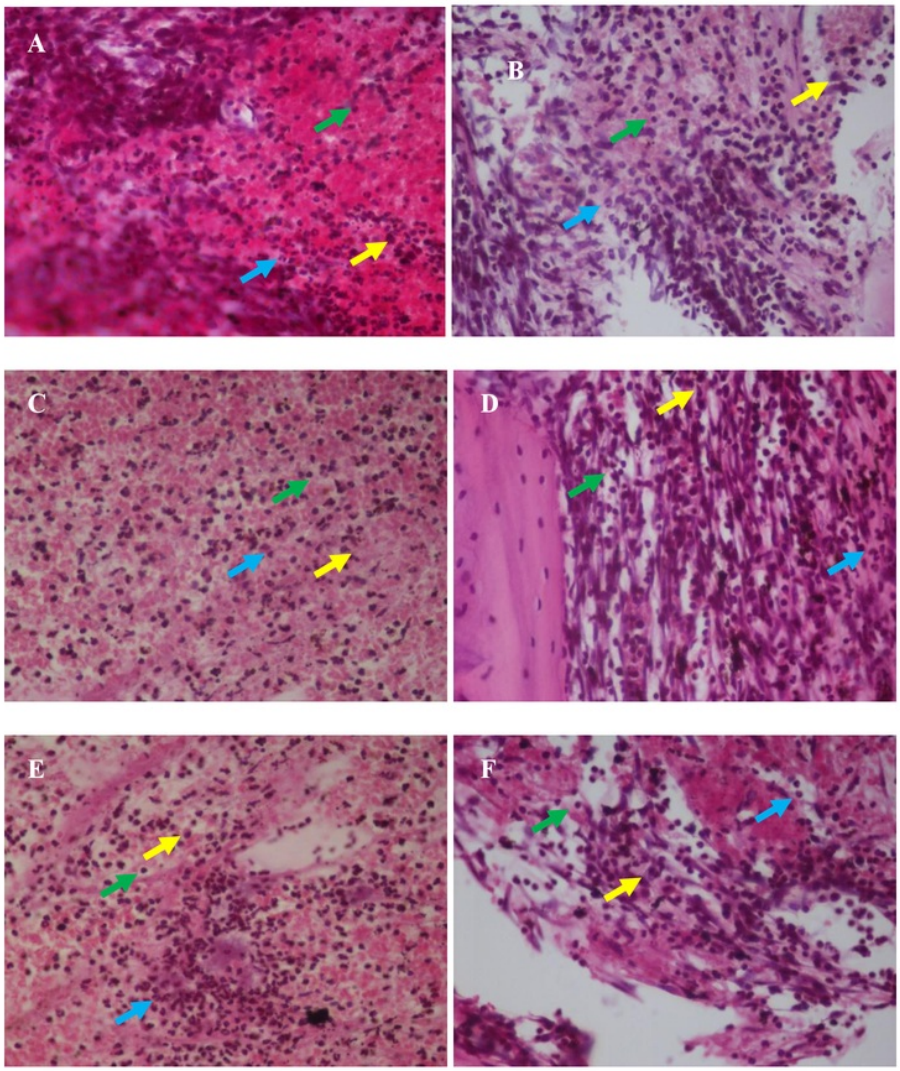

Figure 1. Inflammatory cells in the tooth extraction sockets of Cavia cobaya animals in group KI (A), group PI (B), group KII (C), group PII (D), group KIII (E), and group PIII (F). (blue arrows: PMN, yellow arrows: macrophage cells, green arrows: lymphocytes).

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